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(57) Abstract

The present invention describes a direct interaction between myosin and the cytoplasmic domain of membrane proteins, particularly the phosphorylated cytoplasmic domains of the β–subunit of integrins. The invention provides methods of identifying agents which block integrin binding to myosin, methods of using agents which block integrin binding to myosin to modulate biological and pathological processes, and provides agents that block integrin mediated binding to myosin and thereby modulate related cellular or cellular component movement.

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B. FIELDS SEARCHED

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 97 14432 A (COR THERAPEUTICS INC) 24 April 1997 see the whole document ---	24-31, 33,34
X	WO 95 34641 A (SCRIPPS RESEARCH INST) 21 December 1995 see the whole document ---	24, 26-31, 33,34
X	WO 95 25173 A (SCRIPPS RESEARCH INST) 21 September 1995 see the whole document ---	24, 26-31, 33,34
X	US 5 498 649 A (GUILLOT DAVID G) 12 March 1996 see the whole document ---	24, 26-31, 33,34

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document. with indication.where appropriate. of the relevant passages	Relevant to claim No.
X	LAW DA ET AL: "Outside-in integrin signal transduction. Alpha IIb beta 3-(GP IIb IIIa) tyrosine *phosphorylation* induced by platelet aggregation." J BIOL CHEM, MAY 3 1996, 271 (18) P10811-5, UNITED STATES, XP002062627 see the whole document	24-31, 33,34

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INTERNATIONAL SEARCH REPORT

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WO 9714432 A	24-04-1997	AU 7458696 A	07-05-1997
		CA 2207973 A	24-04-1997
		EP 0801574 A	22-10-1997
WO 9534641 A	21-12-1995	AU 2773595 A	05-01-1996
WO 9525173 A	21-09-1995	US 5523209 A	04-06-1996
		CA 2185446 A	21-09-1995
		EP 0769065 A	23-04-1997
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US 5498649 A	12-03-1996	NONE	

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The present invention describes a direct interaction between myosin and the cytoplasmic domain of membrane proteins, particularly the phosphorylated cytoplasmic domains of the β–subunit of integrins. The invention provides methods of identifying agents which block integrin binding to myosin, methods of using agents which block integrin binding to myosin to modulate biological and pathological processes, and provides agents that block integrin mediated binding to myosin and thereby modulate related cellular or cellular component movement.

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MODULATION OF INTERACTIONS BETWEEN MYOSIN AND INTEGRINS

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 60/031,665, filed November 21, 1996 and U.S. Provisional Application No. 60/042,093, filed March 28, 1997, both of which are hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to the interaction of integrins with components of the cellular cytoskeleton. In particular, a novel interaction between myosin and the cytoplasmic domain of β3 integrin has been discovered and a method of identifying direct interactions between integrin cytoplasmic tails and cellular proteins is described.

BACKGROUND OF THE INVENTION

Integrins are a family of αβ heterodimers that mediate adhesion of cells to extracellular matrix proteins and to other cells (Clark *et al.*, Science (1995) 268:233-239). Integrins also bind to the actin cytoskeleton through a series of intermediate proteins, and thus provide a link between the extracellular matrix and the intracellular cytoskeleton and its associated motile machinery. Such transmembrane linkages are required for cell migration. Many biological responses are dependent at least to some extent upon integrin-mediated adhesion and cell migration, including embryonic development, hemostasis, clot retraction, mitosis, angiogenesis, inflammation, immune response, leukocyte homing and activation, phagocytosis, bone resorption, tumor growth and metastasis, atherosclerosis, restenosis and wound healing.

Members of the integrin family also participate in signal transduction. This is evidenced by an alteration in the adhesive affinity of cell surface integrins in response to

cellular activation, termed inside-out signal transduction. Additionally, effects on intracellular signaling pathways following integrin-mediated adhesion have been observed, termed outside-in signal transduction.

The integrin family consists of 15 related known α subunits (α1, α2, α3, α4, α5, α6, α7, α8, α9, αE, αV, αIIb, αL, αM, and αX) and 8 related known β subunits (β1, β2, β3, β4, β5, β6, β7, and β8). Luscinskas *et al.*, *FASEB J.*, 8: 929-938 (1994). Integrin α and β subunits are known to exist in a variety of pairings as indicated in Figure 1. Integrin ligand specificity is determined by the specific pairing of the α and β subunits, although some redundancy exists as several of the integrins are known to bind the same ligand.

A. Interaction of Integrins with Known Cytoskeletal Proteins

The binding of unmodified α and β subunit cytoplasmic domains of integrins to a variety of cytoskeletal and signaling proteins has been documented. S. Dedhar *et al.*, *Curr. Opin. Cell Biol.* 8:657-669 (1996). Morphological studies have shown that many of these proteins are concentrated in focal adhesions where integrins cluster and bind to both the extracellular matrix and cytoskeletal proteins. I. Knezevic *et al.*, *J. Biol. Chem.* 271(27):16416-16521 (1996).

For example, talin, a 235 kD vinculin and actin binding protein, binds to the cytoplasmic domains of αIIb and β3 in a solid phase binding assay. I. Knezevic *et al.*, *Id.* The binding of α actinin, a 100 kD vinculin binding protein and actin cross-linking protein, to the cytoplasmic domain of β1 and β3 in solid phase binding assays has also been observed. C.A. Otey *et al.*, *J. Biol. Chem.* 268(28);21193-21197 (1993); and C. A. Otey *et al.*, *J. Cell Biol.* 111:721-729 (1990). Binding studies have demonstrated an interaction between the cytoplasmic domain of β1 and tensin, a 215 kD SH2 domain containing vinculin and actin binding protein. S. Lin *et al. Mol. Biol. Cell* 7 Supp. 389a, Abstract 2259 (1996).

Other cytoskeletal related proteins also interact with integrins. Skelemin, a 195 kD myosin and intermediate filament binding protein, binds to the membrane proximal regions of β1 and β3 cytoplasmic domains. K.B. Reddy *et al.*, *Mol. Biol. Cell* 7 Supp. 385A, Abstract 2237 (1995). These authors suggested that skelemin could link myosin and intermediate filaments to β integrins.

Paxillin, a vinculin binding signaling protein also binds to the cytoplasmic domain of the β1 integrin. M.D. Schaller *et al.*, *J. Cell Biol.* 130:1181-1187 (1995). It is not yet known whether the β1-paxillin association is direct or indirect, however paxillin was postulated as being the substrate for and tyrosine phosphorylated by tyrosine kinase pp125 FAK . The actin binding protein filamin has been shown to bind to the cytoplasmic tail of the β2 integrin subunit in vitro and co-immunoprecipitated and co-localized with β2 integrins in vivo. C.P. Sharma *et al.*, *J. Immunol.* 154; 3461-3470 (1995).

A 208 kD integrin binding protein identified as being related to the myosin light chain kinase family of serine/threonine kinases has also been reported. Walker *et al.*, *Mol. Biol.Cell* 7 Supp. 385A, Abstract 2235 (1995). This kinase was said to be part of a complex of proteins including α-actinin and myosin, however, it was unclear whether the kinase associated directly with the cytoplasmic tails of integrins or through a complex of proteins.

Although the cytoskeletal proteins listed above have been shown to interact with cytoplasmic domains of integrin subunits with purified proteins or peptides, it is not known how these interactions occur within cells or how these interactions are regulated. Furthermore, the integrin/cytoskeletal interactions described thus far do not occur in a phosphotyrosine-dependent manner.

B. Tyrosine Phosphorylation of the Cytoplasmic Domain of Integrin β Subunits

Platelet aggregation induced by a number of agonists results in the phosphorylation of tyrosine residues in the β3 cytoplasmic tail. Law *et al.*, *J. Biol. Chem*

271:10811-10815 (1996). In some respects, the phosphorylation of both tyrosine residues was necessary for binding to certain signaling proteins, whereas other signaling proteins bound following monophosphorylation. Furthermore, adhesion to vitronectin by cells transfected with αvβ3 induces a robust tyrosine phosphorylation of the β3 subunit. Blystone *et al.*, *J. Biol. Chem* 271:31458-31462 (1996).

Studies have shown that the sequences of the cytoplasmic domains of β1, β2 and β3 which contain tyrosines are important for normal integrin/cytoskeletal interactions. For example, the substitution of tyrosine 747 by alanine in β3 transfected into CHO cells abolished β3-mediated cell spreading, blocked the recruitment of αIIbβ3 to preestablished adhesion plaques, and decreased the ability of αIIbβ3 to mediate internalization of fibrinogen-coated particles. J. Ylanne et. al., *J. Biol. Chem.*, 270, 9550-9557, (1995).

Additional experiments reported by Ylanne *et al.*, *Id.*, showed further that substitution of alanine for tyrosine 759 decreased cell spreading and the recruitment of αIIbβ3 to adhesion plaques, while deletion of the carboxy terminal pentapeptide that contains this sequence had an even more pronounced effect on the function of the integrin. These authors concluded integrin-mediated cell spreading does not occur because the factors that are absolutely required for integrin-mediated cell spreading cannot bind either the β3 truncated at residue 757 or the integrin with tyrosine 747 of β3 substituted by alanine.

Point mutations in homologous domains in β1- and β2 -containing integrins also modulate function, as these mutations affect integrin-cytoskeletal interactions by reducting focal adhesions, A. A. Reszka et. al., *J. Cell Biol.* 117:1321-1330 (1992), and integrin activation, M. L. Hibbs et. al., *J. Exp. Med.* 174:1227-1238 (1991), respectively. Tyrosine kinases similarly were found to be essential in regulating the cytoskeletal attachment of αIIbβ3. Schoenwaelder *et al.*, *J. Biol. Chem.* 269(51):32479-32487 (1994).

Overall, the interactions between the two tyrosines and the "cell adhesion regulatory domain" or "CARD" of residues 747-762 of the β3 cytoplasmic domain were reported to be essential for regulation of the adhesive function of integrin β3. Liu *et al.*,

PNAS 93:11819-11824 (1996). A 16-amino acid sequence from the CARD inhibited adhesion of HEL and ECV 304 cells to immobilized fibrinogen by competing with intracellular protein-protein interactions that "engage the business end" of the integrin β3 tail. However, the identity of cytoplasmic protein(s) interacting with CARD was said to remain to be established.

C. Myosin

The platelet plasma membrane is coated by a lattice-like structure, known as the membrane skeleton, that is composed of short actin filaments, actin-binding protein, spectrin, vinculin and various other proteins, not all yet identified. Fox *et al., J. Biol. Chem.* 268(34):25973-25984 (1993). On the cytoplasmic side, the skeleton appears to be associated with a network of cytoplasmic actin filaments. The membrane skeleton coats the lipid bilayer and is associated with both extracellular glycoproteins and intracellular cytoskeletal elements. Fox *et al.* suggested that GPIIb-IIIa induces redistribution of components of the membrane skeleton and associated signaling molecules as a step in regulating integrin-induced motile events in platelets.

Myosin is a contractile protein that interacts with actin to produce contraction or movement. The term "myosin" broadly refers to a diverse superfamily, comprised of at least 11 classes, of molecular motors capable of translocating actin filaments or of translocating vesicles or other cargo on fixed actin filaments by. One characteristic of all myosins is their ability to reversibly bind to actin and to hydrolyze MgATP. See Figure 5 and J. R. Sellers and H.V. Goodson, Protein Profile 2:1323-1339 (1995).

All types of myosin that have been purified are multimeric and appear to possess at least three functional domains- a head, neck and tail. The head or motor domain contains nucleotide and actin binding sites and is the most conserved region of the myosin superfamily. The neck domain consists of a long single alpha helical strand from the heavy chain which is stabilized by the binding of light chain subunits. The tail region, which serves to anchor myosin so that it can translocate actin, is the most diverse primary

sequence of all the regions and may serve to anchor certain myosin isoforms to cell or organelle membranes. It has been suggested that myosin clustering within a cell may occur on membranes or on actin filaments themselves. Titus, *Trends in Cell Biology* 7:119 (1997). However, the precise biochemical mechanism of interaction between the myosin tail and cytocellular structures has not heretofore been described.

SUMMARY OF THE INVENTION

The present invention is based in part on the discovery that the contractile protein myosin binds to the cytoplasmic domain of the β3 integrin subunit. Discovery of this association was based on experiments involving the phosphorylation of tyrosine residues in the integrin cytoplasmic domain. Specifically, the integrin-myosin interaction typically is associated with the phosphorylation of one or more tyrosine residues on the integrin cytoplasmic domain. However, interactions that do not include phosphotyrosine residues are not excluded from the scope of the present invention.

Based on this discovery, the present invention provides peptide fragments of the cytoplasmic domains of integrin β subunits, which can be used to identify signaling and cytoskeletal proteins which bind directly to integrin cytoplasmic domains. In a preferred embodiment, the binding of myosin proteins to integrins is detected; however, the binding of integrins to other cytoskeletal proteins or to other signaling partners also are contemplated. These peptide fragments can also be tyrosine phosphorylated (either mono- or di-phosphorylated) to identify proteins that bind to integrin cytoplasmic domains in a phosphorylation-dependent manner. However, such peptides do not necessarily require phosphorylation to be useful in the methods disclosed herein.

The present invention includes methods for identifying an agent which blocks or modulates the interaction of an integrin with myosin comprising the steps of: a) incubating a peptide comprising the phosphorylated cytoplasmic domain of the β subunit of the integrin with myosin and with an agent, and b) determining whether said agent blocks or modulates the binding of myosin to said peptide.

The present invention also provides methods for modulating, reducing, blocking and stimulating the association of an integrin with a cytoskeletal protein. Agents that block integrin/cytoskeletal associations can be used to modulate biological and pathological processes which require an integrin-mediated cytoskeletal attachment. For example, such methods and agents can be used to modulate cellular attachment or adhesion, migration, proliferation and differentiation, and clot retraction. Pathological processes involving such cellular actions include thrombosis, inflammation, tumor metastasis, wound healing and others noted above.

The present invention further provides methods of reducing the severity of pathological processes which require integrin-mediated cytoskeletal association. Since phosphorylation is required for the association of integrins with certain cytoskeletal proteins, agents which block integrin/cytoskeletal association, such as agents which block tyrosine phosphorylation and agents which dephosphorylate phosphorylated tyrosines, and agents that otherwise interfere with integrin-myosin binding can be used in therapeutic methods.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the pairing of α and β integrin subunits.

Figure 2 shows the cytoplasmic domain of various integrin subunits.

Figure 3 shows the binding of β3 peptides to platelet proteins. A) Far Western blots probed with the indicated peptides (1μM). The 200 kD band is recognized specifically by the diphosphorylated β3 peptide. Binding of peptides to the 90 kD band was nonspecific. B) Far Western blots probed with the diphosphorylated β3 peptide (1 μM) and various concentrations of phenylphosphate (0-100 mM). Peptide binding was abolished by 50-100 mM phenylphosphate, indicating that the peptide bound the 200 kD protein in a phosphotyrosine-dependent manner. C) β3 preferentially redistributes to the cytoskeletal fraction following thrombin-induced platelet aggregation.

Figure 4 shows the binding of β3 peptides to purified platelet myosin. A) Anti-myosin immunoblot. Lane 1 contains 10 μg purified platelet myosin and Lane 2 contains 10 μg total platelet lysate. B) Far Western blots probed with the indicated peptides (1μM). Purified platelet myosin is recognized specifically by the diphosphorylated β3 peptide. Lane 1 contains 10 μg purified platelet myosin and Lane 2 contains 10 μg total platelet lysate.

Figure 5A-5B demonstrates that the 200 kD β3 -binding protein activity sedimented with the platelet cytoskeletal fraction.

Figure 6A-6C shows the biding of β3 peptides to purified platelet myosin.

Figure 7A-7D demonstrates the binding of diphosphorylated β3 to purified platelet myosin cleaved by controlled proteolysis .

Figure 8A-8B presents flow cytometric analysis and clot retracting ability of cells expressing mutant β3.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. General Description

The following discussion presents a general description of the invention as well definitions for certain terms used herein.

The presence of phosphotyrosine binding motifs within the cytoplasmic domain of β3 suggests that tyrosine phosphorylation of this integrin tail facilitates the recruitment of phosphotyrosine-binding signaling proteins *Shc* and *Grb2* to the cell membrane. See Philips *et al.* (1996). The present inventors have discovered that tyrosine phosphorylation and dephosphorylation of the β3 integrin tail may, in an analogous manner, regulate integrin association with the cytoskeleton.

II. Specific Embodiments

A. Isolated Peptides.

The present invention provides isolated peptides corresponding to the cytoplasmic domain of the β subunit of an integrin, as well as allelic variants of the integrin cytoplasmic domain and conservative amino acid substitutions of the cytoplasmic domain. The preferred peptide comprises a sequence derived from the cytoplasmic domain of an integrin wherein the tyrosine residues are phosphorylated, typically at domain comprising a NPXY motif (Filardo *et al.* (1995) *J. Cell Biol.* 130:441-50). Such peptides are about 5, 10, 13, 15, 17, 18, 19, 20, 23, 25, 30, 35, 40, 45, 50 or more amino acids in length. The most preferred embodiment consists essentially of the di-phosphorylated peptide corresponding to residues 740-762 of β3 having the following sequence: D-T-A-N-N-P-L-Y(PO3)-K-E-A-T-S-T-F-T-N-I-T-Y(PO3)-R-G-T-COOH (SEQ ID No.).

The peptides of the invention include peptides corresponding the cytoplasmic domain of naturally occurring allelic variants of integrins. This variation results in peptides that have a slightly different amino acid sequence than that specifically recited above. Allelic variants, though possessing a slightly different amino acid sequence than those recited above, will still have the requisite ability to associate with myosin.

As used herein, a peptide is said to be isolated when physical, mechanical or chemical methods are employed to remove the peptide protein from peptides having different primary amino acid sequences or from cellular constituents if the peptides are derived from natural cellular sources or recombinantly expressed in a suitable host cell. A skilled artisan can readily employ standard purification methods to obtain an isolated peptide.

The peptides of the present invention further include peptides having conservative amino acid substitutions compared to a peptide corresponding to the cytoplasmic domain of a naturally occurring integrin. As used herein, a conservative amino acid substitution

refers to alterations in the amino acid sequence that do not adversely affect the ability of the peptide to bind to myosin. A substitution, insertion or deletion is said to adversely affect the peptide when the altered sequence prevents the peptide from associating with myosin. For example, the overall charge, structure or hydrophobic/hydrophilic properties of a peptide can be altered without adversely affecting activity of the peptide. Accordingly, the amino acid sequence of a peptide can be altered, for example to render the peptide more hydrophobic or hydrophilic, without adversely affecting the ability of the peptide to become associated with myosin.

Ordinarily, peptides corresponding to the allelic variants of a given integrin or peptides having conservative amino acid substitutions will have an amino acid sequence having at least 75% amino acid sequence identity with a naturally occurring human integrin cytoplasmic domain, more preferably at least 80%, even more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to such sequences is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the known peptides, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. N-terminal, C-terminal or internal extensions, deletions, or insertions into the peptide sequence shall not be construed as affecting homology.

Thus, the peptides of the present invention include: molecules having the amino acid sequence disclosed as Peptide 1 or peptides corresponding to peptide 1 from β-1, β-2, β-5, β-6, or β-7; fragments thereof having a consecutive sequence of at least about 13, 15, 20 or 23 amino acid residues of Peptide 1 or corresponding peptides from β-1, β-2, β-5, β-6, and β-7, including both tyrosine residues found at positions 747 and 759 of the naturally occurring β3; amino acid sequence variants of such sequences wherein at least one amino acid residue has been inserted N- or C-terminal to, or within, the disclosed sequence; amino acid sequence variants of the disclosed sequence, or their fragments as defined above, that have been substituted by another residue. Contemplated variants

further include those containing predetermined mutations by, *e.g.*, homologous recombination, site-directed or PCR mutagenesis, and the corresponding peptides of other animal species, including but not limited to rabbit, rat, murine, porcine, bovine, ovine, equine and non-human primate species; and derivatives wherein the peptide has been covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (for example a detectable moiety such as an enzyme or radioisotope).

B. rDNA molecules Containing an Integrin Peptide Encoding Nucleic Acid Molecule

The present invention further provides recombinant DNA molecules (rDNAs) that contain a peptide encoding sequence. As used herein, a rDNA molecule is a DNA molecule that has been subjected to molecular manipulation *in situ*. Methods for generating rDNA molecules are well known in the art, for example, see Sambrook *et al., Molecular Cloning* (1989). In the preferred rDNA molecules, a peptide encoding DNA sequence is operably linked to expression control sequences and/or vector sequences.

The choice of vector and/or expression control sequences to which one of the peptide encoding sequences of the present invention is operably linked depends directly, as is well known in the art, on the functional properties desired, e.g., protein expression, and the host cell to be transformed. A vector contemplated by the present invention is at least capable of directing the replication or insertion into the host chromosome, and preferably also expression, of the peptide encoding sequence included in the rDNA molecule.

Expression control elements that are used for regulating the expression of an operably linked peptide encoding sequence are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, and other regulatory elements. Preferably, the inducible promoter is readily controlled, such as being responsive to a nutrient in the host cell's medium.

In one embodiment, the vector containing a peptide encoding nucleic acid molecule will include a prokaryotic replicon, i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, vectors that include a prokaryotic replicon may also include a gene whose expression confers a detectable marker such as a drug resistance. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Vectors that include a prokaryotic replicon can further include a prokaryotic or viral promoter capable of directing the expression (transcription and translation) of the peptide encoding gene sequences in a bacterial host cell, such as *E. coli*. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA), pPL and pKK223 available from Pharmacia, Piscataway, N.J.

Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can also be used to form a rDNA molecules the contains a peptide encoding sequence. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are PSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), the vector pCDM8 described herein, and the like eukaryotic expression vectors.

Eukaryotic cell expression vectors used to construct the rDNA molecules of the present invention may further include a selectable marker that is effective in an eukaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the

gene whose expression results in neomycin resistance, i.e., the neomycin phosphotransferase (*neo*) gene. (Southern *et al.*, *J. Mol. Anal. Genet.* 1:327-341, 1982.) Alternatively, the selectable marker can be present on a separate plasmid, and the two vectors are introduced by co-transfection of the host cell, and selected by culturing in the appropriate drug for the selectable marker.

C. Host Cells Containing an Exogenously Supplied Peptide Encoding Nucleic Acid Molecule

The present invention further provides host cells transformed with a nucleic acid molecule that encodes a peptide of the present invention. The host cell can be either prokaryotic or eukaryotic. Eukaryotic cells useful for expression of a peptide are not limited, so long as the cell line is compatible with cell culture methods and compatible with the propagation of the expression vector and expression of the peptide product. Preferred eukaryotic host cells include, but are not limited to, yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Preferred eukaryotic host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, baby hamster kidney cells (BHK), and the like eukaryotic tissue culture cell lines.

Any prokaryotic host can be used to express a peptide-encoding rDNA molecule. The preferred prokaryotic host is *E. coli*.

Transformation of appropriate cell hosts with a rDNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically employed, see, for example, Cohen *et al.*, *Proc. Natl. Acad. Sci. USA* **69**:2110, 1972; and Maniatis *et al.*, Molecular Cloning, A Laboratory Mammal, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). With regard to transformation of vertebrate cells with vectors containing rDNAs, electroporation,

cationic lipid or salt treatment methods are typically employed, see, for example, Graham *et al.*, *Virol.* **52**:456, 1973; Wigler *et al.*, *Proc. Natl. Acad. Sci. USA* **76**:1373-76, 1979.

Successfully transformed cells, i.e., cells that contain a rDNA molecule of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that described by Southern, *J. Mol. Biol.* **98**:503, 1975, or Berent *et al.*, *Biotech.* **3**:208, 1985 or the proteins produced from the cell assayed via an immunological method.

D. Production of Peptides using a rDNA molecule.

The present invention further provides methods for producing a peptide that uses one of the peptide encoding nucleic acid molecules herein described. In general terms, the production of a recombinant form of a peptide of the invention typically involves the following steps:

First, a nucleic acid molecule is obtained that encodes a peptide. The peptide encoding nucleic acid molecule is then preferably placed in operable linkage with suitable control sequences, as described above, to form an expression unit containing the peptide encoding sequences. The expression unit is used to transform a suitable host and the transformed host is cultured under conditions that allow the production of the peptide. Optionally the peptide is isolated from the medium or from the cells; recovery and purification of the protein may not be necessary in some instances where some impurities may be tolerated.

Each of the foregoing steps can be done in a variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in appropriate hosts. The construction of expression vectors that are operable in a variety of hosts is accomplished using appropriate replicons and control sequences, as set forth above. The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene and were discussed in detail

earlier. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors. A skilled artisan can readily adapt any host/expression system known in the art for use with peptide encoding sequences to produce peptide according to the invention. Recombinant peptide tyrosine residues may be phosphorylated using standard procedures.

E. Methods of Identifying Agents Which Modulate the Interactions Between Myosin and an Integrin.

Another embodiment of the present invention provides methods for identifying agents that reduce or block the association of an integrin with a cytoskeletal protein, such as myosin. Specifically, an integrin or integrin peptide comprising the tyrosin phosphorylated β subunit cytoplasmic domain (such as Peptide 1 disclosed herein) is mixed with a cytoskeletal protein such as myosin in solution or attached to a solid support, in the presence and absence of an agent to be tested. After mixing under conditions that allow association of the integrin or peptide with the cytoskeletal protein, the two mixtures are analyzed and compared to determine if the agent reduced or blocked the association of the integrin with the cytoskeletal protein. Agents that block or reduce the association of an integrin with the cytoskeletal protein will be identified as decreasing the amount of association present in the sample containing the tested agent.

Assays that result in the modulation of integrin association with a cytoskeletal protein such as myosin may utilize any available means to detect or monitor integrin-myosin interactions. Such methods include the direct monitoring of integrin-myosin binding or the monitoring of secondary endpoints such as those exhibited when using permeabilized platelets (Pumiglia *et al.*, (1992) *Biochem J.* 286:441-9). Such methods of detection are widely available as disclosed by Harlow *et al.*, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988. For instance, solid phase assays are widely employed wherein either binding partner is attached to a solid support. In such binding assays, the integrin or peptide derived from the tyrosine phosphorylated β subunit

cytoplasmic domain is typically labeled and incubated with myosin attached to the solid support. Any label may be used, including, but not limited to radioactive, enzymatic, florescent or other dye labels. After suitable incubation, free integrin or peptide is separated from that present in bound form, and the amount of free or uncomplexed label is a measure of the ability of the particular agent to bind to the integrin or integrin peptide or to interfere with the association of the integrin or integrin peptide and myosin. In an alternative format, after suitable incubation and washing, the amount of bound label is used as a measure of the ability of the particular agent to bind to the integrin or integrin peptide or to interfere with the association of the integrin or integrin peptide and myosin.

Another technique useful for screening for agents which modulate the interaction between an integrin or integrin peptide comprising the tyrosine phosphorylated β subunit cytoplasmic domain and a cytoskeletal protein such as myosin is the use of high throughput screening for compounds having suitable binding affinity to the integrin or integrin peptide comprising the β subunit cytoplasmic domain. Such high throughput screening systems are widely available such as those described in European Patent Application 84/03564. Briefly stated, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with the integrin or integrin peptide comprising the tyrosine phosphorylated β subunit cytoplasmic domain and washed. Bound integrin or integrin peptide comprising the β subunit cytoplasmic domain is then detected by methods well known in the art. Purified integrin or integrin peptide comprising the β subunit cytoplasmic domain can also be coated directly onto plates for use in the aforementioned drug screening techniques. In addition, non-neutralizing antibodies can be used to capture the peptide and immobilize it on the solid support.

Alternative assay formats include the use of competitive screening assays in which neutralizing antibodies capable of specifically binding integrin or an integrin peptide comprising the tyrosine phosphorylated β subunit cytoplasmic domain compete with a test compound or agent for binding to the integrin or integrin peptide. Particularly useful

antibodies are those raised against small peptides comprising an integrin β subunit cytoplasmic domain such as Peptide 1 wherein the tyrosine residues have been phosphorylated. In this manner, the antibodies can be used to detect the ability of the agent to competitively bind to the integrin or peptide.

As used herein, an agent is said to reduce or block integrin or integrin peptide/myosin association when the presence of the agent decreases or prevents the integrin or peptide from becoming associated with myosin. One class of agents will reduce or block the association by binding to the integrin or integrin peptide while another class of agents will reduce or block the association by binding to the integrin binding domain of myosin. Other classes of agents include those that block the phosphorylation of the cytoplasmic domain or dephosphorylate the cytoplasmic domain.

Agents that are assayed in the above methods can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences involved in the association of the integrin with myosin. An example of randomly selected agents is the use a chemical library, a peptide combinatorial library or a growth broth of an organism. As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a nonrandom basis which takes into account the sequence of the target site and/or its conformation in connection with the agent's action. As described above, there are two sites of action for agents that block integrin/myosin interaction: the tyrosin phosphorylated cytoplasmic domain of the integrin β subunit or the myosin domain responsible for integrin interaction. Agents can be rationally selected or rationally designed by utilizing the peptide sequences that make up the contact sites of the integrin/myosin complex pair. For example, a rationally selected peptide agent can be a peptide whose amino acid sequence is identical to the tyrosine phosphorylated cytoplasmic domain of the integrin or the integrin contact site on myosin.

The agents of the present invention can be, as examples, peptides, small molecules, vitamin derivatives, as well as carbohydrates. Peptide analogs are commonly

used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or " peptidomimetics" (Fauchere, J. (1986) Adv. Drug Res. 15:29; Veber and Freidinger (1985) TINS p.392; and Evans et al. (1987) J. Med. Chem. 30:1229, which are incorporated herein by reference). A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents of the present invention.

One class of agents of the present invention are peptide agents whose amino acid sequences are chosen based on the amino acid sequence of the tyrosine phosphorylated cytoplasmic domain of the integrin β subunit. In addition to recombinant expression of the desired peptide by the methods set forth above, the peptide agents of the invention can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art.

Another class of agents of the present invention are antibodies immunoreactive with critical positions of the tyrosine phosphorylated cytoplasmic domain of an integrin or with the integrin binding domain of myosin. Antibody agents are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of the integrin β subunit cytoplasmic domain intended to be targeted by the antibodies. Such a peptide is preferably Peptide 1. Critical regions include the contact sites involved in the association of the integrin with myosin, including the antigenic sites which encompass or include the phosphorylated tyrosine residues of Peptide 1.

Antibody agents are prepared by immunizing suitable mammalian hosts in appropriate immunization protocols using the peptide haptens alone, if they are of sufficient length, or, if desired, or if required to enhance immunogenicity, conjugated to suitable carriers. Methods for preparing immunogenic conjugates with carriers such as BSA, KLH, or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be effective; in other

instances linking reagents such as those supplied by Pierce Chemical Co., Rockford, IL, may be desirable to provide accessibility to the hapten. The hapten peptides can be extended at either the amino or carboxy terminus with a Cys residue or interspersed with cysteine residues, for example, to facilitate linking to a carrier. Administration of the immunogens is conducted generally by injection over a suitable time period and with use of suitable adjuvants, as is generally understood in the art. During the immunization schedule, titers of antibodies are taken to determine adequacy of antibody formation.

While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, use of monoclonal preparations is preferred. Immortalized cell lines which secrete the desired monoclonal antibodies may be prepared using the standard method of Kohler and Milstein or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is the peptide hapten or is the integrin or signaling complex itself. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid.

The desired monoclonal antibodies are then recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonals or the polyclonal antisera which contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as the Fab, Fab', of $F(ab')_2$ fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin.

The antibodies or fragments may also be produced, using current technology, by recombinant means. Regions that bind specifically to the desired regions of receptor can also be produced in the context of chimeras with multiple species origin.

The antibodies thus produced are useful not only as modulators of the association of an integrin with myosin, but are also useful in immunoassays for detecting integrin mediated signaling and for the purification of integrin-associated signaling proteins.

F. Uses for Agents which Modulate Myosin-Integrin Interactions.

As provided in the Background section, integrins play important roles in intracellular signaling, cellular attachment, cellular aggregation and cellular migration. Agents that modulate, reduce or block the interactions of an integrin with a cytoskeletal component such as myosin can be used to modulate biological and pathologic processes associated with integrin function and activity.

Both integrins and myosin are known to play a role in mediating cell migration. Mutations in the cytoplasmic domains of the integrin αIIbβ3 which increase ligand affinity to promote cytoskeletal linkages have been shown to decrease the migration rate on a fibrinogen substrate. Huttenlocher *et al., J. Cell Biol.* 134; 1551-1562 (1996). Similar observations have been reported in α4β1-mediated migration in eosinophils. Kuijpers *et al., J. Exp. Med.* 178:279-284 (1993). Eosinophils, although they represent only a minor fraction of circulating leukocytes, are major inflammatory infiltrators in a number of pathological conditions, including asthma, atopic skin reactions, parasitic infestation and some delayed-type hypersensitivity reactions. Kuijpers *et al., J. Exp. Med.* 178:279-284 (1993). β1 integrin deficient mice possess impaired blood cell migration. Hirsch *et al., Nature* 380:171 (1996). Together these studies suggest that integrins are important players in cell migration. Interestingly, β3 integrins in particular are often expressed on highly motile cells, such as melanoma cells. Albeda *et al., Cancer Res.* 50:6757-6764 (1991).

Different myosin classes themselves play different roles in mediating cell motility, and no single myosin is believed to be fully responsible for cell movement. Forces must be generated to extend membrane processes at the front of the cell, and also at the rear to extend the cell body forward. In a simplified hypothesis of cell movement, myosin I is thought to be involved in the extension of pseudopods in the front portion of migrating cells, while myosin II-based contraction occurs in the rear of the cell (Lauffenburger *et al* 1996, 84; 359-369). Integrins are also differentially expressed within the cell. It has been

hypothesized that cell migration is regulated by transient activation of an integrin at the cell front, with subsequent deactivation at the cell rear. Huttenlocher *et al.*, *J. Cell Biol.* 134; 1551-1562 (1996). Continuous reorganization of the cytoskeleton is required for cell motility, since cells change shape as they migrate. The instant invention indicates that it is possible that tyrosine phosphorylation of integrin tails may regulate the association and dissociation of integrin-myosin interactions, and in this way regulate cell motility. Agents that interfere with cell migration based on integrin-myosin interactions may therefore, also control pathologic cell migration such as tumor metastasis or chronic inflammation.

As used herein, a subject can be any mammal, so long as the mammal is in need of modulation of a pathological or biological process mediated by an integrin. The term "mammal" is meant an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

As used herein, a biological or pathological process mediated by an integrin or integrin signaling refers to the wide variety of cellular events in which an integrin binds a cytoskeletal component such as myosin. Examples of biological processes include, but are not limited to, cellular attachment or adhesion to substrates and other cells, cellular aggregation, cellular migration, cell proliferation, and cell differentiation.

As used herein, the phrases "pathological state" or "pathological condition" in reference to the direct interaction of an integrin to myosin includes, but is not limited to thrombosis, inflammation, angiogenesis, tumor metastasis, wound healing (including cutaneous wounds such as burn wounds, donor site wounds from skin transplants and cutaneous, decubitis, venous stasis and diabetic ulcers), acute coronary syndrome, myocardial infarction, unstable angina, refractory angina, occlusive coronary thrombus occurring post-thrombolytic therapy or post-coronary angioplasty, a thrombotically mediated cerebrovascular syndrome, embolic stroke, thrombotic stroke, transient ischemic attacks, venous thrombosis, deep venous thrombosis, pulmonary embolus, coagulopathy, disseminated intravascular coagulation, thrombotic thrombocytopenic

purpura, thromboangiitis obliterans, thrombotic disease associated with heparin-induced thrombocytopenia, thrombotic complications associated with extracorporeal circulation, thrombotic complications associated with instrumentation such as cardiac or other intravascular catheterization, intra-aortic balloon pump, coronary stent or cardiac valve, and conditions requiring the fitting of prosthetic devices.

As used herein, the phrase "cell mobility impairment" is associated with conditions related to sperm motility, inflammation, resistance to infection, immune function, autoimmune disease, wound repair, cancer, immune diseases, and spastic diseases and disorders such as gastrointestinal cramps and contractions related to pregnancy.

Pathological processes refer to a category of biological processes which produce a deleterious effect. For example, thrombosis is the deleterious attachment and aggregation of platelets while metastasis is the deleterious migration and proliferation of tumor cells. These pathological processes can be modulated using agents which reduce or block integrin association to a cytoskeletal component such as myosin.

As used herein, an agent is said to modulate a pathological process when the agent reduces the degree or severity of the process. For example, an agent is said to modulate thrombosis when the agent reduces the attachment or aggregation of platelets.

G. Methods of Treating Pathological Conditions.

The agents of the present invention can be provided alone, or in combination with other agents that modulate a particular pathological process. For example, an agent of the present invention that reduces thrombosis by blocking integrin cytoskeletal association can be administered in combination with other anti-thrombotic agents. As used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

The agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively,

or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The present invention further provides compositions containing one or more agents which block integrin/cytoskeletal association. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise 0.1 to 100 μg/kg body wt. The preferred dosages comprise 0.1 to 10 μg/kg body wt. The most preferred dosages comprise 0.1 to 1 μg/kg body wt.

In addition to the pharmacologically active agent, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

In practicing the methods of this invention, the compounds of this invention may be used alone or in combination, or in combination with other therapeutic or diagnostic agents. In certain preferred embodiments, the compounds of this invention may be coadministered along with other compounds typically prescribed for these conditions according to generally accepted medical practice, such as anticoagulant agents, thrombolytic agents, or other antithrombotics, including platelet aggregation inhibitors, tissue plasminogen activators, urokinase, prourokinase, streptokinase, heparin, aspirin, or warfarin. The compounds of this invention can be utilized *in vivo*, ordinarily in mammals, such as humans, sheep, horses, cattle, pigs, dogs, cats, rats and mice, or *in vitro*.

EXAMPLES

Example 1

Identification of a Specific Interaction Between Platelet Myosin and a Tyrosine-Phosphorylated β3 Cytoplasmic Domain Peptide

Previous studies have established that a subpopulation of αIIbβ3 (GPIIb-IIIa) translocates to the cytoskeleton in aggregated platelets. J.E.B. Fox *et al., J. Biol. Chem.* 268:25973 (1993). Since the extent of αIIbβ3 redistribution to the cytoskeleton was correlated to the extent of platelet aggregation, it was determined whether tyrosine phosphorylation of β3 had any influence on αIIbβ3 redistribution. Platelets were induced to aggregate by the addition of 0.1 U/ml thrombin with stirring and lysed with Triton X-100 lysis buffer. Lysates from either the supernatant fraction of the insoluble pellet were subjected to 2D-gel analysis and the ratio of β3 phosphorylation (with basal phosphorylation subtracted)/total amount of β3 present in the samples as determined by densitometry of five separate experiments. Under the solubilization conditions described in Example 2, approximately 34% (P=0.002) of the total β3 protein associated with the cytoskeletal fraction upon aggregation, in agreement with earlier studies (Figure 3C).

Notably, only about 5% of the αIIbβ3 was found in the cytoskeleton of unstimulated platelets. Densitometry of antiphosphotyrosine immunoblots indicated that approximately 72% (P=0.018) of the tyrosine phosphorylated β3 redistributes to the cytoskeletal fraction. Thus, tyrosine phosphorylated β3 is more than twice as likely to become associated with the cytoskeleton (P=0.018) which may indicate a pivotal role for this β3 modification in linking a ligand-occupied receptor on the surface of aggregated platelets to the cytoskeletal/contractile apparatus within.

Since tyrosine phosphorylation of the cytoplasmic domain of β3 occurs upon platelet aggregation, the present inventors sought to identify cytoskeletal proteins associated specifically with tyrosine-phosphorylated β3 tails. Platelet proteins run on SDS-PAGE were transferred to nitrocellulose and probed with various biotinylated peptides containing sequences of integrin cytoplasmic tails. Using this Far Western approach, direct binding of a peptide corresponding to residues 740-762 of the β3 cytoplasmic domain to a 200 kD protein in platelet lysates was observed (Figure 3A). This binding occurred only when both tyrosine residues (Tyr-747 and Tyr-759) were phosphorylated. Singly phosphorylated β3 peptides, a diphosphorylated β_3 peptide containing a Glanzmann's thrombasthenia mutation (S752P) and an unrelated peptide with similarly spaced phosphotyrosine residues all failed to bind the 200 kD protein in Far Western blots. The abundance and size of this protein suggested that it is myosin heavy chain. Indeed, initial experiments demonstrate that myosin purified from platelets bound the diphosphorylated β3 peptide in a highly specific manner in Far Westerns (Figure 4B). These results suggest that tyrosine phosphorylation of β3 upon platelet aggregation may induce the direct linkage of αIIbβ3 to the contractile protein myosin in the cytoskeletons of aggregated platelets.

Example 2

Confirmation of Myosin as an Integrin-Binding Ligand

A. EXPERIMENTAL PROCEDURES

Platelet Lysate Preparation

Blood from healthy volunteers was drawn on the day of use and washed platelets were prepared as previously described (16) except 0.6 U/ml apyrase and 50 ng/ml prostaglandin 12 (final concentrations) were present in the collecting solution. Before stimulation, the platelets (~4-8X10^8 /ml) were incubated for I hour at 37°C in Tyrodes-HEPES buffer (12 mM NaHC03. 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 10 mM HEPES pH 7.4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$) unless otherwise stated. Platelet samples of 0.5 ml were then stirred at 37°C in a whole blood lumiaggregometer and various agonists and conditions were examined. When platelet lysates were not prepared, 4X non-reducing Laemmli sample buffer containing vanadate (37mM Tris pH 6.8, 11.8% (v/v) glycerol, 2.36% (w/v) SDS, 2mM sodium orthovanadate and 0.002% (w/v) bromophenol blue (final concentration)) was added immediately after aggregation and samples were boiled for 5 minutes.

For two dimensional gel analysis, platelets were lysed immediately after aggregation by the addition of an equal volume of ice cold 2X Triton X-100 lysis buffer (1% (v/v)Triton X-100, 100mM NaCl, 20mM Tris pH 7.0, 2 mM ethylenedinitrilo-tetraacetic acid, 2 mM [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid, 20 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 200 µM leupeptin, 4 mM sodium orthovanadate, 2 mM benzamidine, 50 µg/ml diisopropyl fluorophosphate, 5 mM sodium pyrophosphate (final concentrations)). The lysate was then centrifuged for 6 minutes at 15,000 x g to remove any Triton X-100 insoluble material formed during aggregation. The supernatant was reserved and 100 µl of 2X RIPA was added to the pellet and sonicated for 20 minutes in a Branson 5120 Sonicator to resolubilize the pellet. Non-reducing sample buffer (as

described above) was added to each of the samples (supernatant and resolubilized pellet) and boiled for 5 minutes.

For ligand blot analysis, platelets are lysed in RIPA buffer (I% (w/v) Triton X- 100, 1% (w/v) deoxycholic acid, 0. 1% sodium dodecyl sulfate, 5 mM ethylenedinitrilo-tetraacetic acid, 20 mM Tris pH 7.5, 5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 75 µg/ml leupeptin, 20 µg/ml aprotinin (final concentrations)). For analysis of supernatant and cytoskeletal fractions in ligand blots, platelets were lysed in Triton X-100 buffer (1% (v/v) Triton X-100, 137 mM NaCl, 2 mM ethylenedinitrilo-tetraacetic acid, 20 mM Tris PH 8, 5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 75 µ/ml leupeptin, 20 µ/ml aprotinin (final concentrations)) and cytoplasmic actin filaments were sedimented by centrifugation at 15600 x g for 15 min at 4°C.

Phosphopeptide synthesis

Peptides consisting of cytoplasmic regions of β3 were synthesized by SynPep corporation using solid phase Fmoc chemistry. Peptides were dissolved in water and diluted as needed.

Ligand Blot Analysis

Platelet lysates or myosin proteolytic digests are boiled in Laemmli sample buffer, run on SDS-PAGE and transferred to nitrocellulose. The blots are wet briefly in HEPES blot buffer (HBB) (25 mM HEPES, 25 mM NaCl, 5mM $MgCl_2$ +1 mM dithiothreitol) at 4° C. The transferred proteins are denatured by 6M Guanidine HCI in HBB for ten minutes at 4° C and renatured by two-fold dilution of Guanidine HCI (ten minute incubations each with 3M, 1.5M, 0.75M, 0.38M and 0. 19M and 0 M Guanidine HCI in HBB). The blot is blocked in HBB containing 4% bovine serum albumin overnight at 4° C and probed with 1 µM biotinylated peptide in HBB containing 0.5% bovine serum albumin for 3 hours at

room temperature. After washing in TBS/0.01% NP40 three times at 4° C, peptide-reactive bands are visualized by incubating the blots in horseradish peroxidase-conjugated streptavidin and employing ECL detection.

Purification of Myosin from Human Platelets

Myosin was purified from human platelets as described by J.L. Daniel and J.R. Sellers, *Methods Enzymol.* 215:78-88 (1992).

Solid Phase Binding Assay

Immulon 4 plates were coated with platelet myosin overnight at 4°C. Plates were washed in TBS 0.01% Brij, blocked in 4%BSA/TBS 0.1%Tween (TBST) and incubated with 10 μM biotinylated peptides for 3 hours at room temperature. Plates were incubated with bound peptide was detected with peroxidase substrate (ABTS) at 650 nm using a plate reader (Molecular Devices).

Ligand Blot Protocol

A ligand blot protocol was developed in order to detect direct interactions between phosphorylated integrin cytoplasmic tail peptides and proteins in cellular lysates. See, Crowley *et al.*, *J. Biol. Chem.* 271:1145-1152 (1996). The basic ligand blotting methodology has not previously been applied to the discovery of proteins that bind to integrin cytoplasmic domains, phosphorylated or not. Proteins separated on SDS-PAGE are transferred to nitrocellulose, denatured with Guanidine HCl under reducing conditions and renatured gradually by dilution of Guanidine HCl in reducing conditions as set forth above. The blot is then blocked overnight and probed with biotinylated phosphopeptides, detected with streptavidin HRP and visualized using chemiluminescence.

A phosphorylated peptide corresponding residues 740-762 of β3 was synthesized and coupled to biotin at the amino terminus:

(Peptide 1) Biotin-D-T-A-N-N-P-L-Y(PO3)-K-E-A-T-

S-T-F-T-N-I-T-Y(PO3)-R-G-T-COOH

A control peptide was synthesized with an identical sequence, but unphosphorylated:

(Peptide 2) Biotin-D-T-A-N-N-P-L-Y-K-E-A-T-S-T-F-T-N-I-T-Y-R-G-T-COOH

To examine the physiological importance of protein binding to b3, a doubly phosphorylated peptide which was mutated such that the serine at position 752 was changed to a proline was used. This mutation occurs naturally in a patient with Glanzmann's thrombasthenia and is known to cause a deficiency in platelet aggregation. Y.-P. Chen, et. al., *Proc. Natl. Acad. Sci.*, 89:10169-10173 (1992).

(Peptide 3) Biotin-D-T-A-N-N-P-L-Y(PO3)-K-E-A-T-P-T-F-T-N-I-T-Y(PO3)-R-G-T-COOH

An unrelated doubly phosphorylated peptide based on the ITAM sequence on the T cell receptor zeta chain was also synthesized.

(Peptide 4) Biotin-Q-Q-G-Q-N-Q-L-Y(PO3)-N-E-L-N-L-G-R-R-E-E-Y(PO3)-D-V-L-D-K-R-R-G-R-COOH

Clot Retraction Assays

Clot retraction experiments were performed as described by Chen *et al.* ((1995) *Blood.* 86:2606-15) with minor modifications. In brief, cells were trypsinized, washed twice and resuspended in Dulbecco's Modified Eagle Medium + 25 mM HEPES. 0.5 ml of cell suspension containing 5 x 10^6 cells was mixed with 0. 1 ml fibronectin-depleted plasma in a 12 x 70 mm glass tube treated with Sigmacote. In some experiments, the tyrosine kinase inhibitor genistein was added to 250 μM. Fibrin clots were formed by adding I U/ml thrombin and allowed to retract at 37°C over a 2 to 3 hour period. Extent of clot retraction was measured by removing and weighing the clot.

B. <u>Confirmation of Myosin as the Integrin-Binding Ligand</u>

To determine whether the 200 kD protein plays a role in mediating the binding of phosphorylated β3 to the platelet cytoskeleton, the present inventors determined whether this protein was isolated with the cytoskeletal fraction of Triton X-100- platelet lysates. Platelets were lysed in RIPA buffer and centrifuged into supernatant and pellet fractions as described above. Volume equivalent loads of whole cell lysate, supernatant and pellet fractions (enriched 5 fold relative to whole cell lysate and supernatant loads) were run on 7.5% SDSPAGE, transferred to nitrocellulose and either renatured and probed with 1 μM diphosphorylated β3 peptide (A) or probed with 1 μg/ml anti-myosin antibodies (B).

As shown in Fig. 5A, most of the 200 kD β3 -binding protein activity sedimented with the platelet cytoskeletal fraction, with little diphosphorylated β3 peptide binding activity remained in the supernatant. When the cytoskeletons of thrombin-aggregated platelets were prepared, we also found that the 200 kD protein partitioned almost exclusively to the Triton X-100 insoluble cytoskeleton. Based on these observations, it appeared that the 200 kD diphosphorylated β3-binding protein was an integral component of the platelet cytoskeleton. Together, its cytoskeletal distribution and apparent molecular weight suggested that the 200 kD protein is the heavy chain of platelet myosin. As is illustrated in Fig. 5B, myosin has the same electrophoretic mobility and displayed the same partitioning between supernatant and pellet fractions of Triton-X 100 lysed platelets as the diphosphorylated β3 -binding protein (Fig. 5B).

To confirm that myosin is the diphosphorylated β3-peptide binding protein, myosin was purified from platelets and tested for this activity (Fig 6A). Myosin heavy chain was found to display the same peptide binding specificity as the 200 kD protein in platelet lysates (Fig 4B and 6B): it bound the di-phosphorylated β3 peptide but not unphosphorylated β3, doubly phosphorylated S752P β3, or CD3 zeta ITAM (Peptides 2,3,4, respectively). In Figure 6B, 10 μg of purified platelet myosin (lane 1) and platelet lysate (lane 2) were probed with the diphosphorylated β3 peptide (1μM). Singly phosphorylated β3 peptides were not found to bind the 200 kD protein, in this assay. A similar peptide binding specificity was also observed in solid phase assays, in which

platelet myosin was coated on plates and binding of the various biotinylated peptides detected enzymatically with horseradish peroxidase-conjugated streptavidin.

In order to further establish that the β3 phosphotyrosines were required for the interaction of β3 with myosin, phenylphosphate, a compound known to compete for phosphotyrosine binding sites was used (see Glenney *et al.* (1988) *J Immunol Methods.* 109:277-85). 10 mM phenylphosphate inhibited completely the binding of the diphosphorylated β3 peptide to purified myosin in renatured blots (Fig. 6C), indicating that the β3-myosin interaction was indeed phosphotyrosine-dependent under these binding conditions. To further demonstrate the specificity of binding and to rule out nonspecific charge effects, a β3 peptide with tyrosines 747 and 759 replaced by glutamines also failed to bind purified myosin. Taken together these observations establish the identity of the 200 kD protein as myosin heavy chain and demonstrate that its direct interaction with the β3 cytoplasmic domain peptide is phosphotyrosine-dependent.

Conventional myosin (type II), found in most cell types including platelets, is a hexameric molecule consisting of 2 heavy chains and 2 pairs of light chains. The coiled-coil tail regions of myosin II self-associate to form myosin filaments. Thus, the present inventors have discovered that the doubly phosphorylated β3 integrin tail binds to myosin II purified from platelets. In subsequent experiments, the present inventors have found that the peptide also binds to skeletal muscle myosin.

Example 3

Diphospho-β3 peptide binds the tail region of myosin.

To determine the domain of myosin responsible for binding diphosphorylated β3, purified platelet myosin was cleaved by controlled proteolysis according to the methods of Sellers *et al.* (1988, *Biochemistry.* 27:6977-82) and the resulting fragments were subjected to ligand blot analysis. Cleavage of myosin with papain yields single-headed soluble subfragment-1 (S1) and an insoluble coiled-coil rod fragment, while cleavage

with chymotrypsin results in double-headed heavy meromyosin and coiled-coil light meromyosin. Digests using these proteases were separated by SDS-PAGE after removing 10 µl aliquots into 1 mM PMSF at the designated times indicated in the Figure 7. Coomasie blue staining gave the expected hydrolytic products (Figs. 7A,C). Ligand blot analysis (Figs. 7B,D) showed that the doubly phosphorylated β3 peptide bound to the rod portion of the papain digest Fig. 7B) and the light meromyosin fragment in chymotrypsin-digested myosin (Fig. 7D). Diphosphorylated β3 did not bind to intact heavy meromyosin. Neither the unphosphorylated β3 peptide nor the diphosphorylated S752P β3 peptide bound to any of these myosin fragments. Since the chymotryptic light meromyosin fragment and the rod portion generated by papain cleavage both contain overlapping sequences within the coiled-coil tail region of myosin, the data indicate that this region is responsible for binding the diphosphorylated cytoplasmic β3 domain.

Example 4

The tyrosine residues within the β3 cytoplasmic domain are important for β3-dependent clot retraction in transfected CHO cells.

Given the above *in vitro* observation that the cytoplasmic domain of β3 interacts with myosin in a phosphotyrosine-dependent manner, the role of the β3 cytoplasmic domain tyrosine residues in a β3-dependent contractile process was assessed using a well established CHO cell expression system (see O'Toole *et al.* (1990) *Cell Regul.* 1:883-93 and Ylanne *et al.* (1993) *J Cell Biol.* 122:223-33). CHO cells, transfected with a β3 cDNA in which both cytoplasmic tyrosine residues were mutated to phenylalanines, were examined for their ability to retract fibrin clots. It has previously been shown that CHO cells, transfected with wild-type β3, can express the β3 on the cell surface in conjunction with endogenous α_v chains (Ylanne *et al.* (1993) *J Cell Biol.* 122:223-33). These $\alpha_v\beta3$-bearing CHO cells are then able to retract clots, a process thought to mimic $\alpha_{IIb}\beta3$-dependent clot retraction in platelets. When CHO cells expressing the Y(747,759)F mutation were used in this assay, the weights of the clots generated were 60 ± 12.5%

(P=0.0024) more than that observed with the wild-type transfectants (see Fig. 8B). This increase in clot weight directly reflects a decreased ability to retract clots. The difference in clot retraction observed between the wild-type and Y(747,759)F transfectants was not due to differences in $\alpha_v\beta3$ expression levels as confirmed by FACS analysis of these cells using the $\alpha_v\beta3$-specific antibody, LM609 (Fig. 8A).

In Figure 8A, mock-transfected CHO cells (i) or cells transfected with wild-type β3 (ii) or Y(747,759)F β3 (iii) were incubated with control mouse IgG *(thick line)* or LM609 (thin line) and analyzed by flow cytometry. The median channels for LM609-staining were 3.59 for mock transfectant, 108.41 for wild-type β3, and 113.42 for Y(747,749)F β3.

The tyrosine kinase inhibitor, herbimycin A, was used as another means of assaying the role of β3 phosporylation in myosin binding because of its ability to inhibit platelet-mediated clot retraction. Treatment of wild-type β3 CHO cell transfectants with the broad spectrum tyrosine kinase inhibitor, genistein, also inhibited clot retraction by approximately 40% (data not shown). These data suggest that protein tyrosine phosphorylation is important for successful clot retraction.

Although previous investigators have determined, using CHO cells co-transfected with β3 and α_{IIb}, that clot retraction observed in nucleated cells appears to be largely $\alpha_v\beta3$ rather than $\alpha_{IIb}\beta3$-dependent, other investigators have recently questioned this result, instead finding that $\alpha_{IIb}\beta3$ appears also to be important in this process (Lyman *et al.* (1997) *J. Biol Chem.* 272:22538-47). The data presented in Fig. 8 used CHO cells transfected only with β3 cDNAs. To assess whether the presence of $\alpha_{IIb}\beta3$ would affect the results, similar experiments were performed with CHO cells that had been co-transfected with the mutant and wild-type β3 cDNA as well as α_{IIb}. These cells expressed both $\alpha_v\beta3$ and $\alpha_{IIb}\beta3$ their surface and essentially the same results were obtained: the cells bearing Y747,759F β3 showed about a 50% decrease in their ability to retract fibrin clots when compared to those cells expressing wild-type β3. Thus, regardless of the contributions of $\alpha_v\beta3$ versus $\alpha_{IIb}\beta3$ to the clot retraction process, the data indicate that the tyrosine residues within β3 play a critical role in this event.

Example 5

Other Myosin Binding Interactions

The presence of novel classes of myosin in platelets has not been formally investigated. However, the invention includes all types and subtypes of myosin, not limited to those expressed in platelets. By use of the foregoing techniques, it can readily be confirmed whether (and which) integrin β subunits also bind other or novel myosin isoforms, and whether (and which) myosins bind other phosphorylated integrin β subunits or the cytoplasmic domains of other proteins that share the NPXY motif. Myosin-integrin binding itself could represent a general mechanism for connecting adhesive proteins to the contractile apparatus in platelets and other cells.

As set forth above, diphosphorylated β3 integrin tail peptide binds to the coiled-coil tail region of myosin. Members of the myosin II class are found in all animal cells and share many common structural properties. Based on sequence and functional differences myosins are divided into the following subclasses: 1) skeletal and cardiac muscle myosin II and 2) smooth muscle and nonmuscle myosin II. There are at least two genes for nonmuscle myosin II, namely IIA and IIB, that are differentially expressed in cells. Myosin IIA is the subtype found predominantly in platelets, lymphocytes, neutrophils and brush border enterocytes, while neuronal tissue mainly expressly myosin IIB. We have also discovered that the diphosphorylated β3 peptide binds to skeletal muscle myosin II. Thus it is likely that the integrin tail binds to a common structural element in the myosin II family, and not just to platelet myosin.

As noted, other integrin cytoplasmic tails possess NPXY motifs (*i.e.*, β1, β5, β6 and β7) and therefore would be envisaged to bind to members of the myosin superfamily in a similar manner. Similarly, other proteins that have this motif would be expected to bind to members of the myosin superfamily and related proteins. This binding may or may not require the involvement of phosphorylated tyrosine residues. For example, Zambrano *et*

al, J. Biol. Chem 272:6399-6405 (1997) discusses a phosphorylation independent interaction between the Fe65 protein and the β-amyloid precursor protein which has this motif.

Example 6

Methods of Identifying Agents Which Modulate the Interaction Between Myosin and Integrins

Agents which modulate the interaction between myosin and integrins are isolated by incubating a peptide comprising the phosphorylated cytoplasmic domain of the β subunit of an integrin with myosin in the presence of the agent to be tested. Agents which inhibit or interfere with the interaction(s) between the peptide and myosin are identified by the modulation, decrease or inhibition in peptide-myosin interactions. Any means available to detect agent mediated quantitative or qualitative differences in the interaction between the peptide and myosin can be used. For instance, the ligand blot procedure set forth above is employed. In this format, platelet lysates, purified myosin or myosin proteolytic digests are boiled in Laemmli sample buffer, run on SDS-PAGE and transferred to nitrocellulose. The blots are wet briefly in HEPES blot buffer (HBB) (25 mM HEPES, 25 mM NaCl, 5mM $MgCl_2$ +1 mM dithiothreitol) at 4° C. The transferred proteins are denatured by 6M Guanidine HCI in HBB for ten minutes at 4° C and renatured by two-fold dilution of Guanidine HCI (ten minute incubations each with 3M, 1.5M, 0.75M, 0.38M and 0. 19M and 0 M Guanidine HCI in HBB). The blot is blocked in HBB containing 4% bovine serum albumin overnight at 4° C and probed with 1 μM biotinylated peptide in HBB containing 0.5% bovine serum albumin in the presence or absence of thee agent to be tested for 3 hours at room temperature. After washing in TBS/0.01% NP40 three times at 4° C, peptide-reactive bands are visualized by incubating the blots in horseradish peroxidase-conjugated streptavidin and employing ECL detection. Modulation of the interaction between an integrin and myosin is detected by a decrease or

increase in the detected binding of the biotinylated peptide to the myosin or myosin fragment in the presence of the agent.

In another format, Immulon 4 plates are coated with platelet myosin overnight at 4°C. Plates were washed in TBS 0.01% Brij, blocked in 4%BSA/TBS 0.1%Tween (TBST) and incubated with 10 μM biotinylated peptide for 3 hours at room temperature. Plates were incubated with bound peptide was detected with peroxidase substrate (ABTS) at 650 nm using a plate reader (Molecular Devices). Modulation of the interaction between an integrin and myosin is detected by a decrease or increase in the detected binding of the biotinylated peptide to the myosin or myosin fragment in the presence of the agent.

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents, applications and publications referred to in the application are hereby incorporated by reference in their entirety. Furthermore, application serial No. 08/753,038, filed November 18, 1996, is hereby incorporated by reference in its entirety.

WHAT IS CLAIMED:

1. A method of modulating the direct interaction of an integrin with myosin in a cell, comprising the step of administering to the cell an agent that modulates the interaction of myosin and integrin in the cell.

2. The method of claim 1, wherein the level of interaction is increased by increasing the binding of integrin to myosin in the cell.

3. The method of claim 1, wherein the level of interaction is decreased by decreasing the binding of integrin to myosin in the cell.

4. A method for blocking the direct interaction of an integrin with myosin, comprising the step of contacting an integrin with an agent that blocks the binding of the integrin to myosin.

5. The method of claim 4 wherein said agent blocks the binding of the integrin to myosin by selectively and competitively binding to the phosphorylated cytoplasmic domain of the β subunit of said integrin.

6. The method of claim 4 wherein said agent blocks the binding of the integrin to myosin by selectively and competitively binding to the myosin.

7. The method of claim 4 wherein said agent blocks the phosphorylation of the cytoplasmic domain of the β subunit of the integrin.

8. The method of any of claims 1 to 7 wherein said agent is a peptidomimetic.

9. The method of any of claims 1 to 7 wherein said integrin comprises a β subunit selected from the group consisting of the β-1, β-2, β-3, β-5, β-6 and β-7 subunits.

10. The method of claim 9 wherein the integrin comprises a β-3 integrin.

11. The method of claim 8 wherein said agent dephosphorylates the cytoplasmic domain of the β subunit of the integrin.

12. The method of claim 9 wherein said agent is a phosphorylated peptide.

13. The method of claim 12 wherein said phosphorylated peptide is derived from the cytoplasmic domain of the β subunit of an integrin.

14. The method of claim 12 wherein said phosphorylated peptide comprises the amino acid sequence of Peptide 1 together with fragments and variants thereof.

15. The method of claim 9 wherein said blocking reduces, for an integrin expressing cell, an integrin-mediated activity selected from the group consisting of cellular aggregation, attachment, adhesion, migration, proliferation and differentiation.

16. The method of claim 9 wherein said blocking reduces cellular aggregation of an integrin expressing cell.

17. A method for reducing the severity of pathological state mediated by direct integrin attachment to myosin comprising the method of claim 9.

18. The method of claim 17 wherein the pathological state is selected from the group consisting of thrombosis, inflammation, angiogenesis, tumor metastasis and wounds.

19. The method of claim 18 wherein the wound is selected from the group consisting of cutaneous wounds such as burn wounds, donor site wounds from skin transplants and
cutaneous, decubitis, venous stasis and diabetic ulcers.

20. A method for identifying an agent which modulates the interaction of an integrin with myosin comprising the steps of:

a) incubating a peptide comprising the phosphorylated cytoplasmic domain of the β subunit of the integrin with myosin and with an agent, and

b) determining whether said agent modulates the binding of myosin to said peptide.

21. The method of claim 20 wherein said peptide comprising the phosphorylated cytoplasmic domain of the β subunit of said integrin is selected from the group consisting of the β-1, β-2, β-3, β-5, β-6, and β-7 subunit.

22. The method of claim 21 wherein said integrin comprises a β-3 integrin.

23. The method of claim 20 wherein said peptide comprises the amino acid sequence of Peptide 1, together with fragments and variants thereof.

24. An agent consisting essentially of the amino acid sequence of Peptide 1, together with fragments and variants that remain effective to modulate the interaction of an integrin with myosin and peptidomimetics thereof.

25. The agent of claim 24 wherein one or more of the tyrosine residues in said amino acid sequence is irreversibly phosphorylated.

26. A method for treating pathological conditions, comprising:

the administration of the agent selected according to claim 20,

wherein the condition is selected from the group consisting of acute coronary syndrome, myocardial infarction, unstable angina, refractory angina, occlusive coronary thrombus occurring post-thrombolytic therapy or post-coronary angioplasty, a thrombotically mediated cerebrovascular syndrome, embolic stroke, thrombotic stroke, transient ischemic attacks, venous thrombosis, deep venous thrombosis, pulmonary embolus, coagulopathy, disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, thromboangiitis obliterans, thrombotic disease associated with heparin-induced thrombocytopenia, thrombotic complications associated with extracorporeal circulation, thrombotic complications associated with instrumentation such as cardiac or other intravascular catheterization, intra-aortic balloon pump, coronary stent or cardiac valve, and conditions requiring the fitting of prosthetic devices.

27. The method of claim 26, wherein the agent is the agent of any of claims 1 to 7.

28. The method of claim 26, wherein a thrombus contracts as a result of the peptide administered having a modulatory effect on the binding of $\alpha IIb\beta_3$ to myosin.

29. A method for treating pathological conditions, comprising:

the administration of the agent selected according to claim 20,

wherein the condition is selected from the group consisting of disorders associated with impaired cell motility.

30. The method of claim 29, wherein the agent is the agent of any of claims 1 to 7.

31. The method of claim 29, wherein the cell motility impairment is associated with a condition relating and selected from the group consisting of sperm motility, inflammation, resistance to infection, immune function, autoimmune disease, wound repair, cancer, immune diseases, and spastic diseases and disorders such as gastrointestinal cramps and contractions related to pregnancy.

32. The method of claim 4, wherein said blocking blocks the interaction between myosin and the NPXY motif of the integrin cytoplasmic domain.

33. A method of inhibiting or reversing reperfusion injury in a subject comprising the step of administering to a subject in need thereof an effective amount of an agent that decreases the interaction of myosin and integrin.

34. The method of any one of claims 1-7, 24, 26, 28 29, 31, 32 or 33, wherein the agent is coadminstered with one or more other active ingredients.

FIG. 1

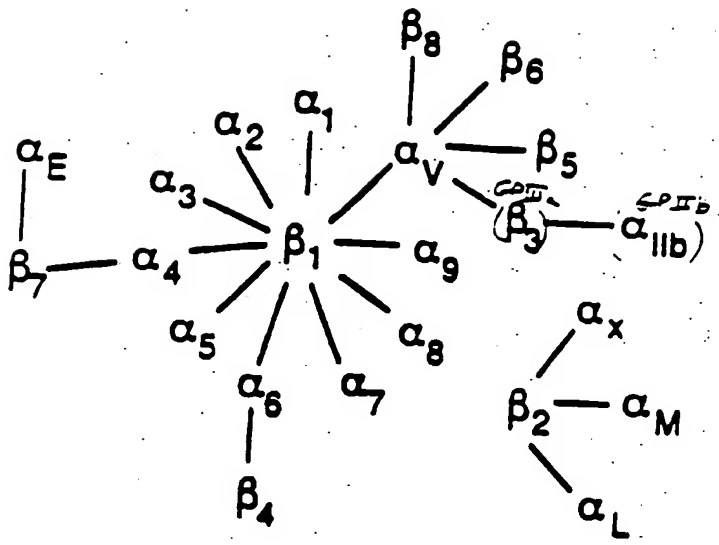
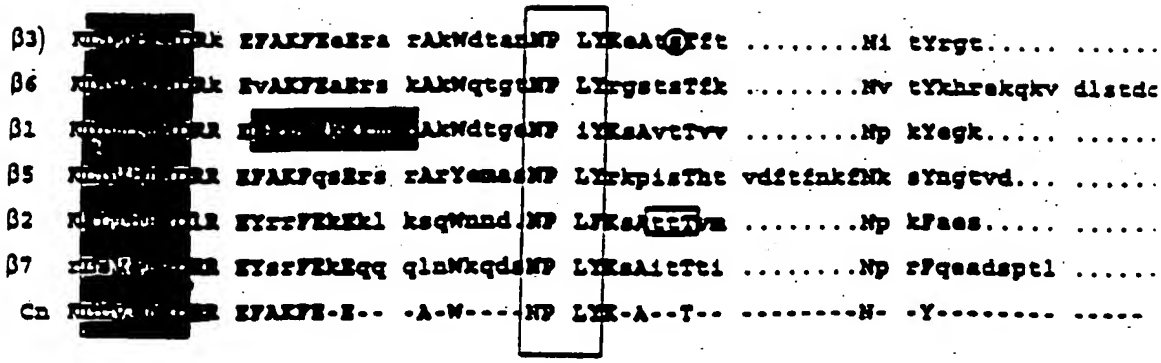
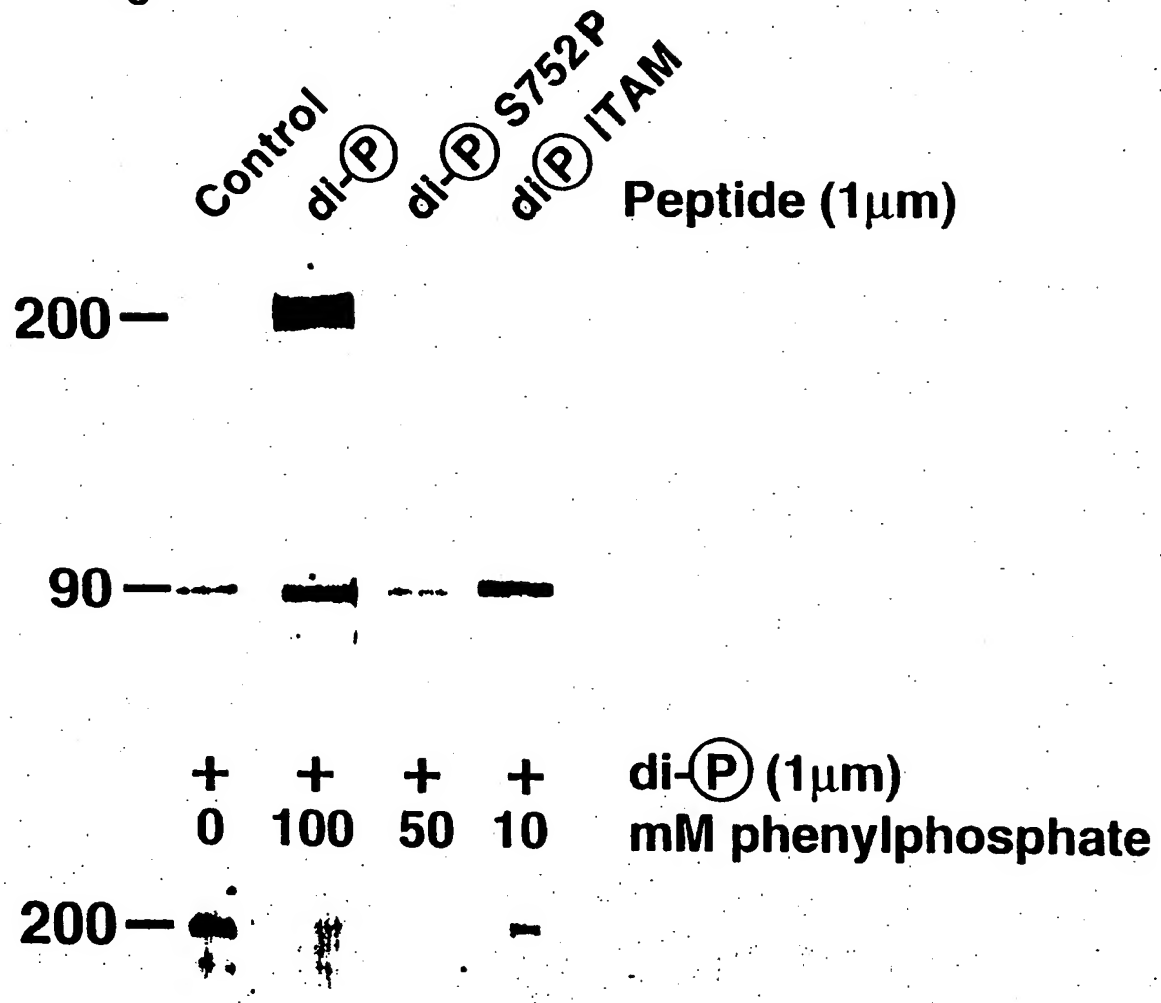


FIG. 2



: 7 Alignment of β subunit cytoplasmic domains. The cytoplasmic domains of human β subunits, with the exception of β_4, we PILEUP and displayed using PRETTY programs of the UWGCG package (61). Consensus residues were defined by their presence if the sequences and are displayed in *capital letters*. Note the conservation of the sequence in β_1(Leu^{717}-Asp^{723}) (*dark gray box* uts. The α actinin-binding decapeptide (56) of β_1 is surrounded by the *hatched box*. A *light gray box* surrounds the conserved NPXY implicated in particle phagocytosis (51) in β_2. Ser^{752} is *circled*: substitution of a Pro at this position disrupts bidirectional integrin The TTT sequence involved in the adhesive function of β_1 is *boxed* (62).

Binding of β_3 Peptides to Platelet Proteins



FIGS. 3A - 3B

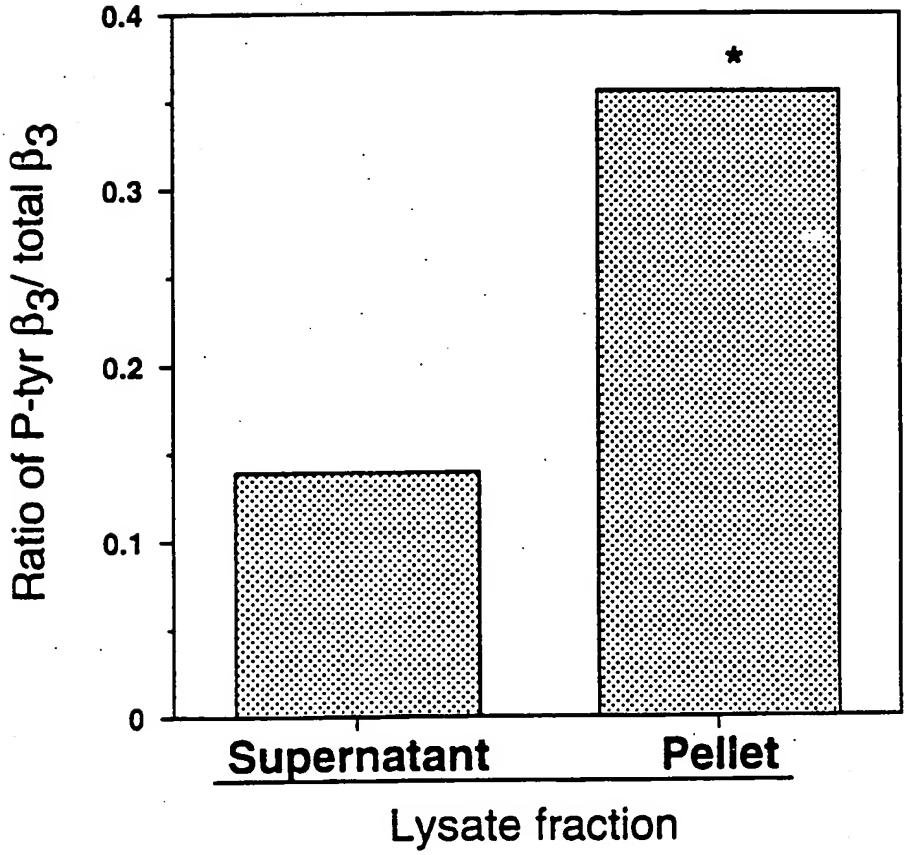
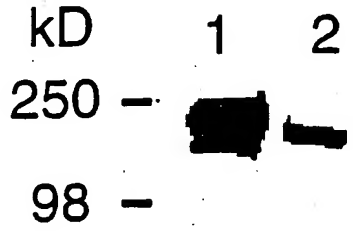

0.4
0.3
0.2
0.1
0
Ratio of P-tyr β3/ total β3
*
Supernatant
Pellet
Lysate fraction

FIG. 3C

Binding of β_3 peptides to purified platelet myosin

A) Western Blot



B) Far Western Blots

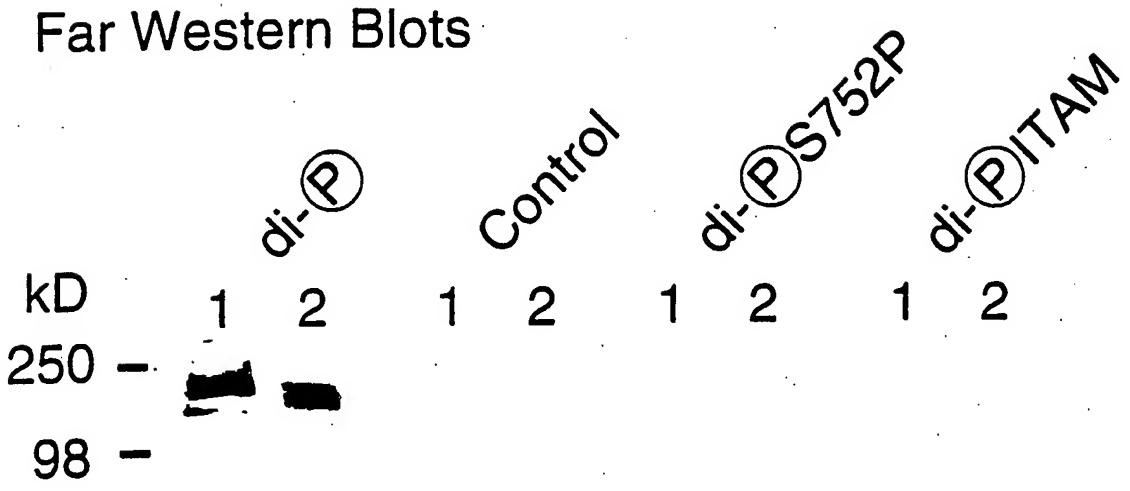
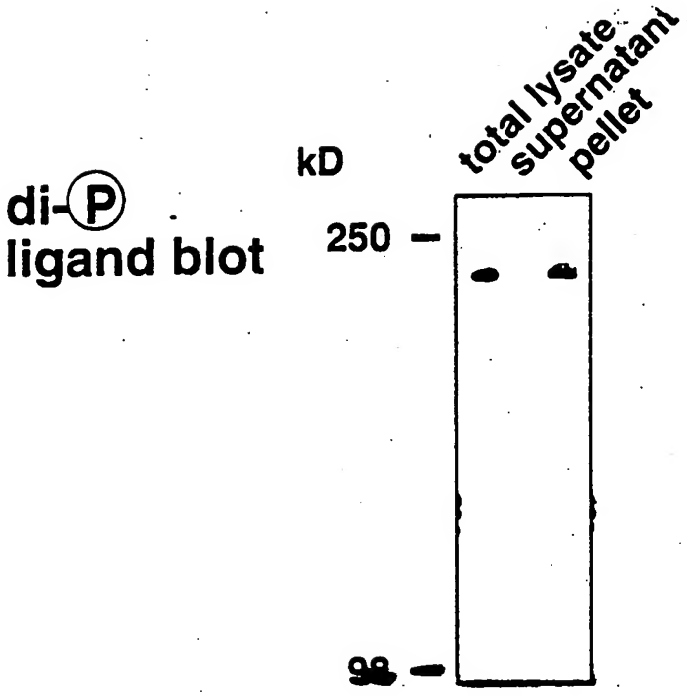
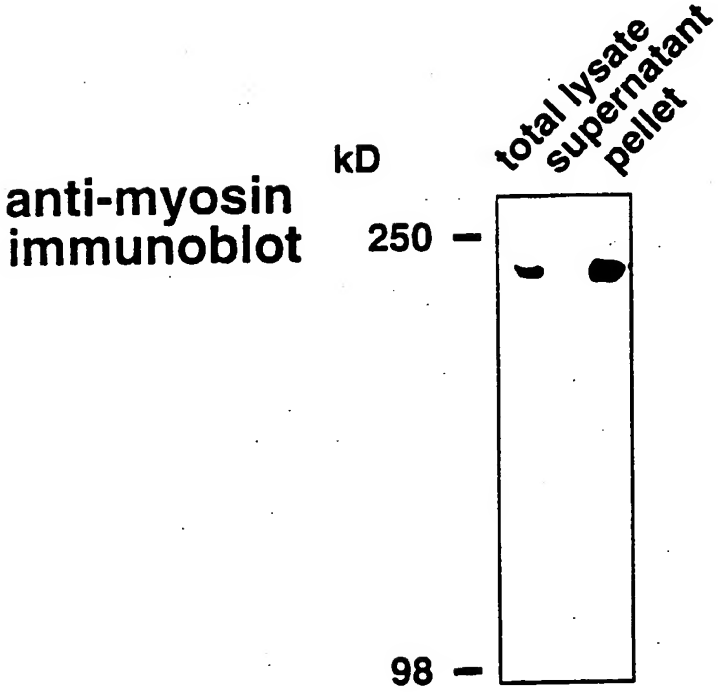


FIG. 4

A)

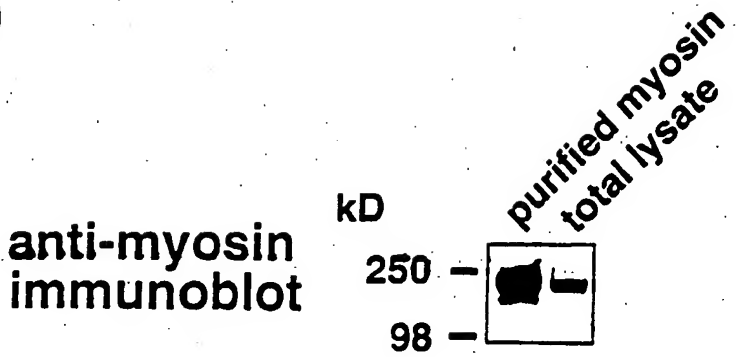

di-Ⓟ
ligand blot
kD
total lysate
supernatant
pellet
250
98

B)

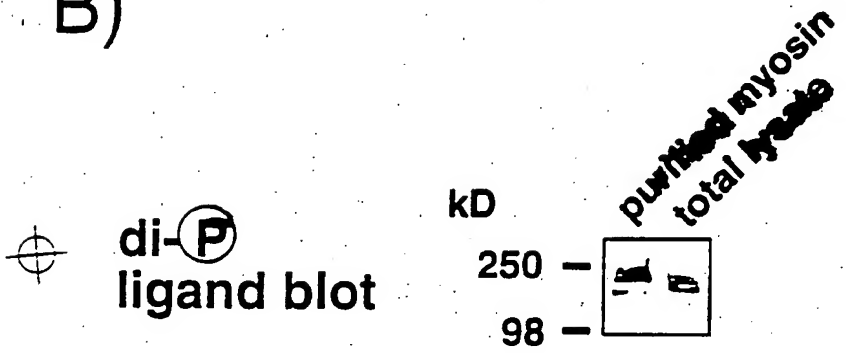

anti-myosin
immunoblot
kD
total lysate
supernatant
pellet
250
98

FIGS. 5A - 5B

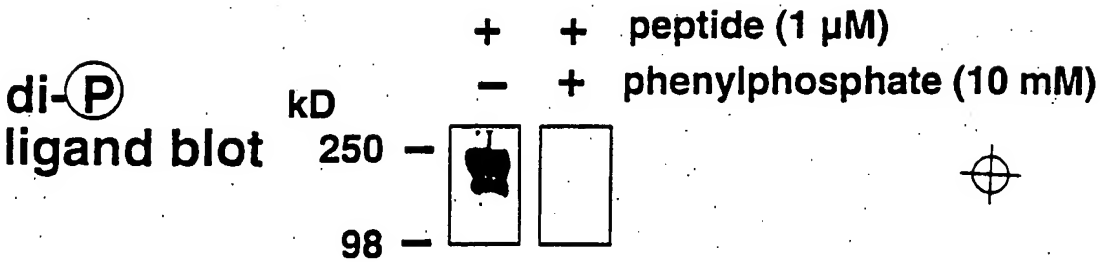
A)



B)



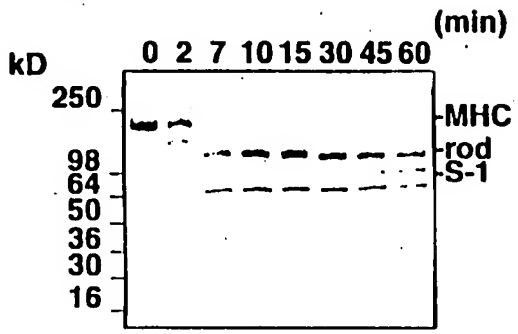
C)



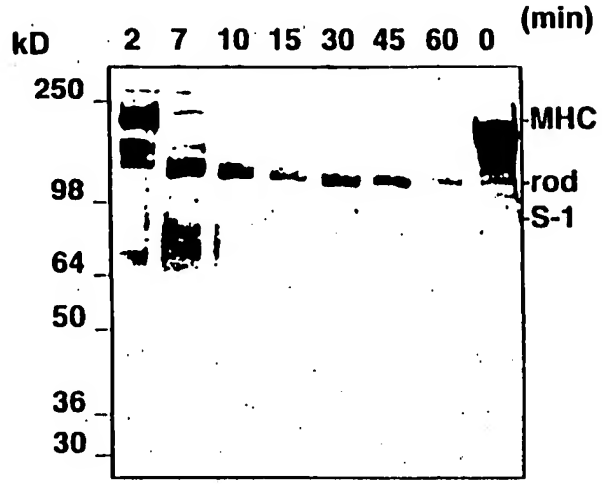
FIGS. 6A - 6C

PAPAIN DIGEST

A) SDS-PAGE

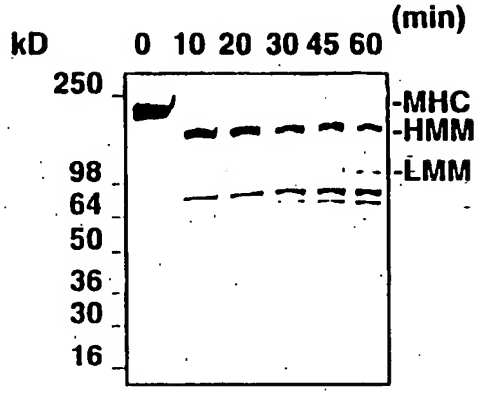


B) Ligand Blot



CHYMOTRYPSIN DIGEST

C) SDS-PAGE



D) Ligand Blot

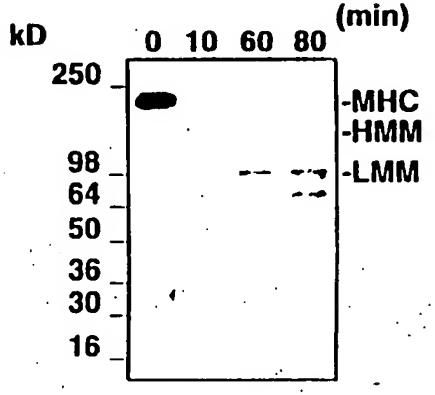
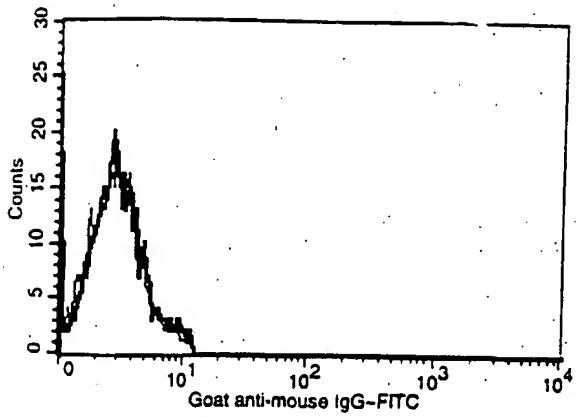


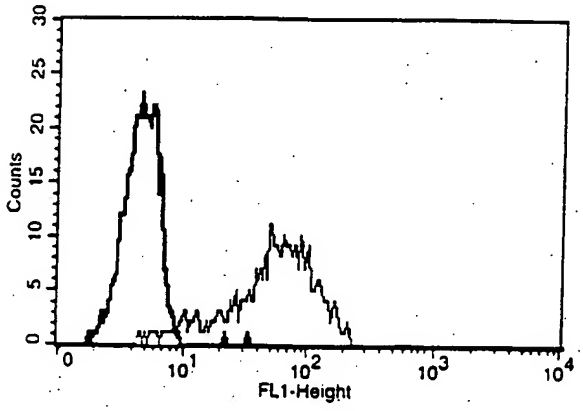
FIG. 7

A

i

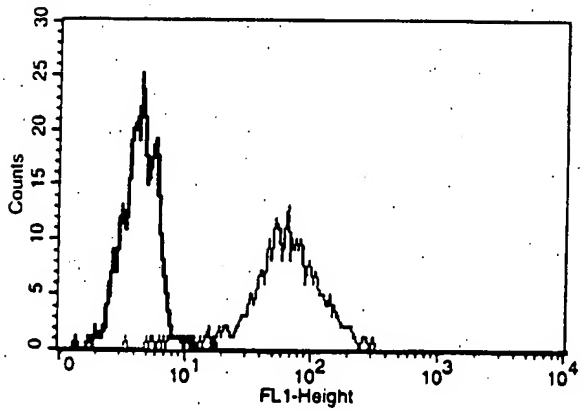


ii



CELL NUMBER

iii



FLUORESCENCE CHANNEL

FIG. 8A

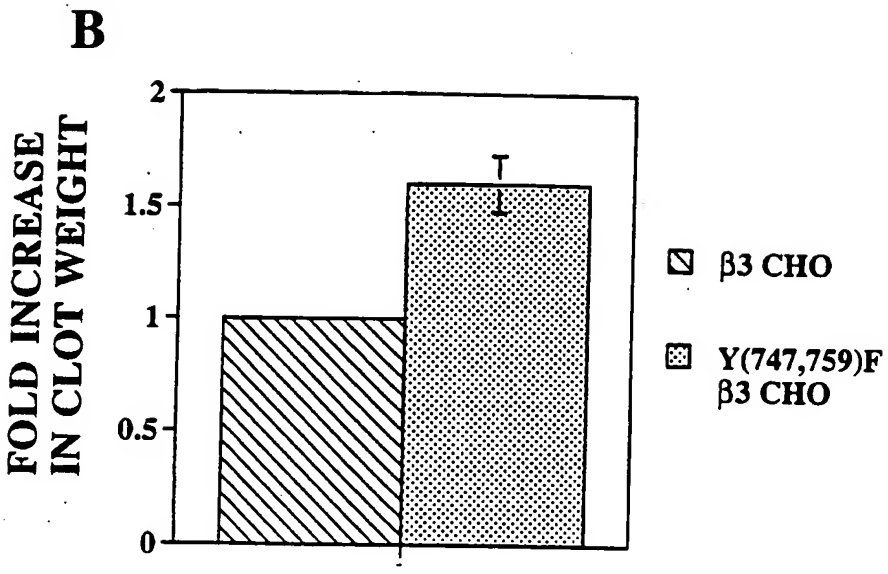

B
FOLD INCREASE IN CLOT WEIGHT
2
1.5
1
0.5
0
β3 CHO
Y(747,759)F β3 CHO

FIG. 8B